IMMOBILIZED BIOCATALYTIC ENZYMES IN ELECTRODEIONIZATION (EDI)

CONTRACTUAL ORIGIN OF THE INVENTION

The United States Government has rights in this invention pursuant to Contract No. W-31-109-ENG-38 between the U.S. Department of Energy (DOE) and The University of Chicago representing Argonne National Laboratory.

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 1.78(a)(3) of provisional application Serial No. 60/423,925 filed November 5, 2002, the entire contents of which are incorporated herein by reference.

I. Overview.

Electrodeionization (EDI) technology utilizes loose ion exchange resins used for water purification. In these commercially available systems, water is fed through an EDI stack, and dilute charged particles are removed. Because the resin is loosely held in the stack, sealing is difficult and leakage can be a problem. ANL technology uses an ion exchange resin EDI system to remove charged impurities (e.g., salts) from commercially valuable products, whereby leakage becomes a significant concern. Thus, a new resin EDI system was designed to prevent leakage. The resins are packed tightly together in a "resin wafer" used to contain and separate charged molecules in EDI.

Chemical reactors utilize chemical reactions and catalysts to convert starting material to commercially valuable products, where separations technologies are required for the downstream capture of the chemical product, to separate out the product from by-products and starting material. Bioreactors utilize biological components to convert starting material to commercially valuable products and also require downstream separations to purify product from starting material. Conventionally, the production and the separation occur in separate process components. ANL technology has combined these two process components, the EDI separation with the biological production to create the "Separative Bioreactor". The ANL Separative Bioreactor is a modified EDI technology composed of the patented resin wafer, disclosed in U.S. Patent application serial number 10/213,721 filed August 6, 2002, the disclosure of which is incorporated by reference which non-specifically contains the biological catalysts (enzymes or micro-organisms), sandwiched between ion-exchange membranes placed within an electrical

current in an electrodialysis stack, which maintains pH control and separates and purifies charged products efficiently.

The resin wafer provided the opportunity to design an in-situ enzyme capture system for specific immobilization of the enzyme within the resin wafer. The resin wafer in this invention is composed of two kinds of resins, one is the ion-exchange resins (i.e., same-charge or mixed-charge ion-exchange resins) and the other is the enzyme capture-resin. The combination of the capture-resins and the ion-exchange resins in the resin wafer will be referred to as the "*charged capture resin*" (Note: the term referring to the "capture-resin" alone without ion-exchange resins is hyphenated, whereas "*charged capture resin*" is in italics un-hyphenated). The *charged capture resin* provides the opportunity for genetically engineered tagged-enzymes to be specifically immobilized within the separative bioreactor. The *charged capture resin* separative bioreactor allows for (1) specific enzyme immobilization, (2) *in situ* enzyme stripping and regeneration, and (3) normal enzymatic activity rates.

II. Utility of Invention

(1) Tagged-enzymes provide specific immobilization and prevent enzyme inactivation.

Conventionally, enzymes are immobilized on a variety of solid surface matrices in bioreactors using weak (ionic) or strong (covalent) attachment technologies. These non-discriminatory global attachment mechanisms often block enzyme active sites, disrupt enzyme structure, and prevent enzyme motion, which result in a significant loss of specific activity typically leaving the enzymes ~10%-30% active.

Glucose Fructose Oxidoreductase (GFOR) is the model enzyme used in this invention. GFOR carries out two reactions, converting glucose to gluconic acid while converting fructose to sorbitol. A genetically added tag on GFOR is an advantage for immobilization in that the enzyme attachment takes place at one specific site on the enzyme (either the N-terminus or C-terminus), leaving the enzyme structure, activity, and active site intact. Several methods of GFOR immobilization have been employed using various tags and capture-resins intercalated within the resin wafer: (1) Histidine-tagged GFOR (His-GFOR) immobilized to Ni^{++}-resin (Nickel-resin), (2) His-GFOR immobilized to Co^{++}-resin (Cobalt-resin), and (3) Biotinylated avidin-tagged GFOR (Biotin~Avi-GFOR) immobilized to avidin-resin. By this tagged-enzyme method, GFOR activity remained at ~100% when immobilized

(2) Tagged-enzymes provide *in situ* enzyme regeneration.

Most immobilized enzymes have lifetimes ranging from 1-2 months while membranes have lifetimes ranging from 1-2 years. Enzymes become the limiting factor in the length of bioreactor lifetimes. Regenerating the enzyme-immobilized-ion-exchange resin wafer becomes a dilemma, because the resins must be completely removed and the whole stack disassembled in order to add back active enzymes, then the whole stack must be reassembled. Thus, if the enzyme can be immobilized and regenerated ten times *in situ* on the same matrix, this significantly reduces the labor and cost to replace inactive enzyme. In addition, because of the conventional covalent immobilization of enzymes, stripping off inactive enzymes damages the potential enzyme-binding sites on the matrix. Enzyme regeneration in the separative bioreactor is efficient with tagged enzymes because a histidine homologue (imidazole) non-

destructively flushes the immobilized His-tagged enzymes from the Co^{++} and Ni^{++} capture-resin. Likewise, biotin displaces the biotinylated Avi-tagged enzyme from the avidin capture-resin. Once a tagged-enzyme loses activity, it is flushed from the resin wafer, and within the same resin wafer, the capture-resin is ready to immobilize the next batch of active tagged-enzyme. Engineering the enzyme with a tag provides the ability to regenerate enzymes *in situ* within the separative bioreactor without having to disassemble the stack, replace the resin wafer, and reassemble the stack.

III. DATA

A. DATA for Binding Capacity and Regeneration

The capture-resin contains a specified binding capacity. Once the capture-resin is intercalated amongst the ion-exchange resins in the resin wafer, it is possible that the other resins and the binding material (latex) may obstruct potential binding sites. Thus, the binding capacities of the *charged capture resins* in the resin wafer were tested.

Another advantage that the tagged-enzyme provides is the opportunity for *in situ* enzyme purification. Because enzymes are purified from microorganisms, obtaining a batch of enzymes requires a prolonged purification process requiring several chromatographic columns before enzymes may be immobilized to a membrane. The capture-resin in the resin wafer provides the advantage of one-step purification and immobilization of the enzyme directly in the separative bioreactor system, bypassing the need for purifying the enzyme before immobilization. Immobilization of tagged-GFOR from whole cells (lysate) was determined.

i. His-GFOR Ni-resin Immobilization & Regeneration

Experiment

Initial studies tested the ability to purify tagged-enzyme directly in the three-resin wafer in columns. His-GFOR in whole cell lysate was added to one of the three (1) cationic exchange resin plus Ni-resin in the resin wafer (*charged capture resin*), (2) cationic exchange resin in a resin wafer without Ni-resin (cation exchange resin), and (3) Ni-only resin (2.5 ml Ni resin in 1st and last). We tested the ability of the three-resin wafer in a column to immobilize His-GFOR from cell lysates. The amount of enzyme that could be stripped from the *charged capture resin* was also determined. Fresh cell lysate containing His-GFOR was added to the stripped resin-wafer for a second immobilization, then stripped again.

2.5 ml of Ni-only resin has a binding capacity for 6.25 mg – 12.5 mg of His-GFOR.

	Immobilized	Strip	Second immobilization	Strip 2
Ni-only resin	12 mg	~12 mg	Not tested	Not tested
charged capture resin	10 mg	~7.6 mg	Out of ~3 mg added to wafer, all 3 mg immobilized	~3 mg
cation exchange resin	0.5 mg	Not detectable	Out of ~3mg added to wafer, ~0.28 mg immobilized	Not detectable

Conclusion: The binding capacity of the capture-resin is specific and at full capacity when intercalated with the ion exchange resin and latex binding material in the resin wafer.

Conclusions: The Promega avidin-resin retained 25-50% of it's binding capacity when in the resin wafer. The avidin-resin is ~40% smaller than Ni-resin, such that clumping may block binding sites. In addition, the latex material that was used to form the *charged capture resin* wafer was not yet optimized. Thus, the binding capacity can be increased. But proof-of-concept is shown for Avi-GFOR immobilization and stripping.

B. DATA for Immobilized Enzyme Activity

It was determined whether GFOR immobilized by the *charged capture resin* method (with and without current) retained 100% activity.

i. Immobilized His-GFOR Activity

Experiment

His-GFOR in whole cell lysate was added to a *Ni-resin + cationic exchange resin* to form a *charged capture resin* wafer (2.5 ml Ni resin) in a column (no electrical current). Immobilized His-GFOR remained active only 12 hours without buffer.

Experiment (2)

~8-9 mg of His-GFOR was immobilized on ~50% Ni-resin/~35% anion exchange resin ~15% cation exchange resin to form a *charged capture resin wafer* in a stack (20 ml Ni resin) in the EDI process (under electrical current). Normally at room temperature the specific activity of 1g of non-immobilized His-GFOR in buffered solution produces 2 g of gluconate/min. The specific activity of 1 g of immobilized His-GFOR without buffer in the EDI process was normal and also produced 2 g of gluconate/min. Figure 1 shows

Experiment (2)

10 g of pure His-GFOR was added to ~50% Ni-resin/~35% anionic exchange resin/~15% cationic exchange resin to form a *charged capture resin wafer* in a stack (20 ml Ni-resin) in the EDI process (under electrical current). Pure His-GFOR was fed directly to the resin wafer.

20 ml of Ni-only resin has a binding capacity of 50-100 mg.

All 10 g of His-GFOR bound to the capture-resin in the *charged capture resin* wafer.

ii. His-GFOR Cobalt-resin Immobilization

As histidine has an affinity for Nickel, histidine also has an affinity for cobalt (Co^{++}). Co^{++}-resin was also used to immobilize His-GFOR. Binding capacity of the Co^{++}-resin was similar to the nickel-resin. Co^{++}-resin is an option because this metal does not leach off of the resin, like Nickel does in the Ni-resin.

iii. Biotin~Avi-GFOR Avidin-resin Immobilization & Stripping

Avi-GFOR was expressed and biotinylated *in vivo* by commercially available protein expression microorganism systems (e.g. BL21(DE3)pLysS, Qiagen or AVB101 Avidity, Colorado). Biotion~Avi-GFOR in cell lysate was added to 0.5 ml avidin-resin intercalated with ion exchange resin to form a *charged avidin capture resin* wafer in a column.

Promega Avidin-resin binding capacity is 0.4 – 0.8 mg per ml resin.

	Immobilized	Strip
charged avidin capture resin	0.2 mg	0.1 mg at 40°C for 30 min.
charged avidin capture resin	0.2 mg	0.15 mg at 40°C for 1 hr.

the production of gluconic acid using EDI stack by immobilized His-GFOR on the Ni-Resin Wafer. Activity was tested for 3 days.

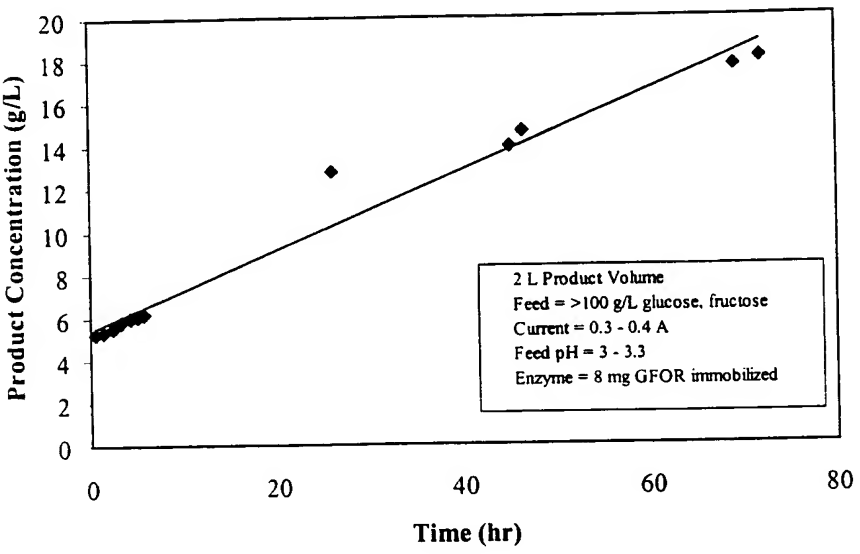

Figure 1. Gluconic Acid Production from Immobilized GFOR in EDI stack.

At pH 5, organic acid concentration is <1 mg/L. Thus to maintain pH an extremely high flow-rate is needed when the enzyme in aqueous solution. This leads to a lower productivity than could typically be obtained in fermentation. Our work shows that although we were able to feed pH at a value of >4.8, our productivity was only 0.02 g/L-feed hr (gluconic acid production) when the enzyme was circulated through in aqueous solution. However, when we immobilized enzymes using the nickel-histidine tag of the above-described technique, we had a productivity of 0.4 g/L-feed hr (gluconic acid) at pH 3.3 (enzyme inactive at this pH in aqueous solution). Not only is this a 20X enhancement in productivity, but it is also a demonstration that immobilized EDI separation allows enzymes to work at pH levels where they otherwise would be inactive.

Conclusions: Immobilized His-GFOR was 100% active in the EDI process for an extended time without buffer. Immobilized enzymes remain 100% active, but without the EDI process to maintain pH and separate inhibitory products, the lifetime of activity is limited.

ii. Immobilized Avi-GFOR Activity

Immobilized Avi-GFOR specific activity was normal when immobilized on *charged capture resin*, not under electrical current.

IV. Summary.

Using tagged enzymes in the separative bioreactor process offers several advantages. The tagged-enzyme capture-resin method was shown to not damage enzyme structure during immobilization as shown by the immobilized enzymes retaining normal activity. The capture-resin within the resin wafer provided a site-specific immobilization technique within the separative bioreactor. Finally, the tagged-enzyme was stripped *in situ*, and fresh tagged-enzyme added *in situ*, without having to disassemble and reassemble the separative bioreactor system.

Although specific examples ion-exchange resins are disclosed herein, the invention encompasses all the ion-exchange resins in the previously filed U.S. patent application serial number 10/213,721 filed August 6, 2002, the entire disclosure of which is incorporated herein by reference, including the PCT application WO 01/12292 and the Terada et al. U.S. patent 6,071,397 referenced therein.

Although specific examples of enzyme capture resins are disclosed, any resin system used to purify genetically tagged enzymes are useful in the present invention. The his tag and the biotin tag are both examples of the more generic 'affinity tags' which are engineered to be part of the protein of interest and are used as capture tags in the process of affinity chromatography

Common for all types of Affinity Chromatography is that an *affinity ligand (i.e. the avidin or the Ni2+)* specific for a binding site *(e.g. the his tag or biotin) on* the target molecule, is coupled to an inert chromatography matrix. (*This 'affinity matrix' is what the third resin is in our wafer)*

Under suitable binding conditions this affinity matrix will bind molecules according to its specificity only. All other sample components will pass through the medium unadsorbed. (*This is how they put the molecule of interest or the enzyme onto the wafer)*

After a 'stripping' or flushing wash step the adsorbed molecules are released and eluted by changing the conditions towards dissociation or by adding an excess of a substance that displaces the target molecule from the affinity ligand. (*This is how they remove the exhausted molecule from the affinity matrix. They then change conditions to the binding conditions above and attach new molecules to the matrix in the wafer)*

Three groups of properties of the target molecule are used in the design of an affinity matrix. There are matrices that exploit specific binding properties based on biological activity for example- Enzyme activity, Receptor binding, Antibody binding, Avidin/Biotin interaction etc. There can also be naturally occurring prosthetic groups in the molecule of interest or enzyme e.g. polysaccharides etc. There are also bioengineered

molecules equipped with an affinity tag like:- Glutathione-S-Transferase (GST) or- Oligo histidine (his tag) etc.

There are two types of affinity ligand in use to-day. Mono-specific affinity ligands are closely related to the structure of the molecule of interest and as such are not often available commercially. In this case the researcher must make his own affinity matrix through the use of well established coupling chemistries e.g. CNBr , Thiol Exchange etc.

Group specific affinity ligands have a much wider applicability and are therefore commercially available. Examples of common group ligands are in the following table.

Group-specific ligand	Specificity
Protein A	Fc region of IgG
Protein G	Fc region of IgG
Concanavalin A	Glucopyranosyl and Mannopyranosyl groups
Cibacron Blue	Broad range of enzymes, serum albumin
Procion Red	NADP+ dependent enzymes
Lysine	Plasminogen, ribosomal RNA
Arginine	Serine proteases
Benzamidine	Serine proteases
Calmodulin	Proteins regulated by calmodulin
Heparin	Coagulation factors, lipoproteins, lipases, hormones, steroid receptors, protein synthesis factors, Nucleic acid-binding enzymes
Transition metal ions	Proteins and peptides which contain accessible Histidine

Stripping solutions are generally specific to the resin or material being stripped, such as up to biomolecule denaturation temperatures for avidin resin.

1-10 mM desthiobiotin for biotinylated tags.

1-50 millimolar biotin or pH 2.0 for biotinylated tags.

5-10 M guanidine - HCl, pH 1.5 for avidin resin.

1 mM to 1 M imidazole for histidine tags.

1 mM to 1M EGTA (Ethylene glycol-O,O' -bis-[2-amino-ethyl]-N,N,N',N',-tetraacetic acid) for CBP tags.

100% ethylene glycol or low salt conditions (<5mM) for CBD tags.

low concentrations of dithiothreitol (1-10 mM) for CCXXCC tags.

chitin binding domain tags using intein amino acid sequences are released from chitin-resin by thiols of free cysteine.

BACKGROUND OF THE INVENTION

The present invention relates to electrodionization (EDI) and more particularly, a method for treating fluids via electrodionization.

Electrodionization (EDI), also known as electrochemical ion-exchange, is an advanced ion-exchange technology that combines the advantages of ion-exchange and electrodialysis. In electrodionization processes, ion-exchange resins are sequestered in dilute feed compartments to increase the ionic conductivity, so that even with a very dilute ionic feed (10^{-1} N), a stable operation with higher flux and lower energy consumption than electrodialysis, becomes possible. The electric power also splits the water molecule (H_2O) in to H+ and OH- ions and the resins are thus regenerated while the ions are removed.

EDI technology presently is used to make deionized water for boiler feed and high purity industrial water applications. There are also many other potential uses of such technology for organic process streams in a variety of industries. One problem encountered in the production of organics such as organic acids and amines is pH control. For example, efficient biocatalytic production of acids and amines is often limited to a narrow pH range for optimal productivity. The present invention provides very accurate pH control using EDI technology , which in combination with previous work at Argonne National Laboratory (ANL) relating to EDI cell design, results in superior production, separation and concentration of a wide variety of organics.

Ion exchange beads that are commonly used for EDI applications may contain strongly acidic resins containing for instance sulfonic acid groups, or strongly basic resins containing for instance quaternary ammonium groups. Other resins such as those with weakly acidic resins for instance carboxylic acid groups or weakly basic resins for instance amines groups are also used when required, and according to the invention may be mixed as required. These resin beads are cross-linked with polymers usually styrene - divinyl benzene or acrylates. The resins can be gel type or macro-reticular type. Usually equivalent mixtures of cationic and anionic resins have been used in the EDI compartments. For specialized applications one type of resin or adsorbent beads mixed with ion-exchange resins may be used. A device useful in the practice of the present invention is disclosed in PCT Application WO 01/12292, the entire disclosure of which is incorporated by reference. Another publication disclosing resins applicable to EDI processes is the Terada et al. U.S. patent 6,071,397 issued June 6, 2000, the entire disclosure of which is also incorporated by reference. Also applicable to the present invention are U.S. patent no. 6,464,880 issued October 15, 2002 and U.S. application serial no. 10/213,721 filed August 6, 2002, the entire disclosure of which is also incorporated herein by reference.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method of controlling the pH or capturing ionic organics in an EDI process.

Another object of the present invention is to provide a method of transferring ionizable organics while controlling the pH in an EDI process and device.

Yet another object of the invention is to provide an economical method for continually producing organic acids and/or amines while controlling the pH to within one pH unit.

Briefly, the invention utilizes the previously mentioned ANL developments including ion-exchange resins having cation-exchange moieties and anion-exchange moieties immobilized relative to each other conferring ion-conductivity and liquid permeability to the material in an EDI stack continuously to produce, separate and concentrate dilute organics while controlling the pH thereof.

The invention also provides a method of controlling the pH of a fluid during electrodionization by adjusting one or more of the resin content in the wafers, adjusting the ratio of strong or weak acid resins to strong or weak base resins in the wafers, the concentration and amount of ionizable organic material flowing through the EDI stack and the current applied to the EDI stack while regenerating the resin, *in situ*.

The invention consists of certain novel features and a combination of parts hereinafter fully described, illustrated in the accompanying drawings, and particularly pointed out in the appended claims, it being understood that various changes in the details may be made without departing from the spirit, or sacrificing any of the advantages of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of an EDI process illustrating the present invention;

FIG. 2 is a graphical representation showing the relationship between the solution pH of an organic acid and the cation ratio in the resin mixture;

FIG. 3 is a graphical representation of the relationship between the concentration of gluconate and the time of the enzymatic reaction producing gluconic acid;

FIG. 4 is a graphical representation of the relationship between the concentration of lactic acid and the time of fermentation producing lactic acid with the pH controlled by the process of the subject invention;

FIG. 5 is a graphical representation of the relationship between the pH in a fermentation reactor as a function of time in a controlled EDI reactor using the method of the subject invention; and

FIG. 6 is a graphical representation of the relationship between pH and current utilization in an EDI process incorporating the present invention.

DETAILED DESCRIPTION OF THE INVENTION

A general overview of a suitable electrodionization device 10 is illustrated as numeral 10 in FIG. 1. An important feature of the device 10 is the porous, immobilized ion-exchange material 12 which facilitates rapid deployment of ionic constituents out of a diluate feed 14.

The wafer material, discussed in the previously mentioned and incorporated PCT application WO 01/12292, is positional intermediate, a cation exchange membrane 16 and an anion exchange membrane 18, the entire triad therefore comprising a reaction chamber 15. A bipolar membrane 19 may be positioned intermediate the terminal anion membrane 18 and the anode 22. A means for facilitating ion transport through the EDI stack is employed. For example, an electrical potential imparted via opposing electrodes 20, 22 (cathode and anode, respectively) provides the gradient to facilitate ion transfer out of the diluate feed 14, and into the respective product chambers 17, each defined by adjacent cathode membrane 16 and anion membrane 18.

The porous immobilized ion-exchange material previously described in PCT application WO _01/12292 may be formed into wafers 12 having relatively uniform thicknesses of between approximately 2 and 6 millimeters. The wafers 12 are suitably porous with between 20 percent and 60 percent porosity so that a liquid will flow through with minimal resistance and the resin beads should be uniformly dispersed in close proximity to each other. "Porosity" is construed herein as the macroscopic void space that can be filled by a liquid.

Referring to FIG. 1, typically the diluate stream or feed 14 consisting of an ionizable fluid such as an ionizable organic wherein the organic may be present in extremely dilute solutions such as 10^{-3} normal. The diluate feed 14 enters into the EDI device 10 and migrates upwardly (it permeates upwardly), as is well known in the art, contacting the various ion-exchange resins in the wafers 12. At the same time, the feed

stream 14 is subjected to the effect of an anion exchange membrane 18 and a cation exchange membrane 16 under the potential applied across the device 10. As illustrated in FIG. 1, the cations migrate through the cation exchange membranes 16 and anions migrate through the anion exchange membranes 18 transferring at least some of the ionizable organic from the ion conducting fluid inside the reaction chambers 15 into the product chambers 17. Simultaneously, ion exchange resins in the wafers 12 become depleted; however, under the influence of the potential across the anode 22 and cathode 20, the ionizable fluid splits into a proton and a negative ion, while the proton regenerates the ion exchange resins in the wafers 12 the negative ions from the ionizable fluid reacts with the protons produced as the ionizable organic disassociates. It is the association of the positive portion of the ionizable organic and the negative portion of the split ion conducting fluid which controls the pH of the material within the reaction chamber 15. A crucial aspect of the present invention is pH control.

An important feature of the present invention is the ability to produce continuously various organic acids or organic amines or other ionizable organics while maintaining close pH control notwithstanding that production of certain organic materials causes the pH either to rise automatically or to lower automatically. It is the maintenance of the pH control within one or within one-half pH unit that allows the continuous production of the organics.

When ions pass from the reaction chambers 15 into the product chambers 17, the ions reassociate to form a concentrated product stream. The product stream is

concentrated with respect to the concentration of the product in the diluate feed 14. Accordingly, the present invention may be used to concentrate ionic materials present in an ion conducting fluid. Moreover, the pH control in the reaction chambers 15 may be maintained either in acidic ranges or in basic ranges. For instance, the pH control by the present invention has been maintained in the range of from 3 to 7, 3 to 5, and 5 to 7. More particularly, within the above-stated ranges, the pH has been controlled within one-half pH unit in any one of the aforementioned ranges. Further, the pH may be controlled in the reaction chambers 15 from 7 to 11 and more particularly, from 8 to 10 and within one or one-half pH unit. An ionizable organic acid and/or organic amine may be produced continuously from an ion conducting aqueous fluid having a substance, as previously described, which is capable of producing the ionizable organic acid and/or ionizable organic amine. The variables which control the pH within the reaction chambers 15 include the electrical potential applied across the stack cathode 20 and anode 22, adjustment of the resin content in the wafers 12, adjustment of the ratio and types of the ion exchange resins in the wafers 12, the amount of the substance producing the ionizable organic acids and/or organic amines in the ionizable fluid flowing through the EDI device, the flow rate of the diluate feed 14, and various combinations of these variables.

As is known in the art, the ion exchange resins applicable to the present invention and most particularly to the wafers 12 may be selected from strong acid resins, weak acid resins, strong base resins and weak base resins, all as previously described.

Referring now to FIG. 2 of the present invention there is shown the relationship between the variation in the cation ratio in the resin mixture and the pH for a weak acid-strong base resin mixture and for a strong acid-strong base resin mixture. The figure and the data on which it is based show that the pH is affected by the content of the resins in the wafers 12. FIG. 3 shows the enzymatic reaction and separation of a gluconic acid in the EDI device 10 of the present invention. In the data illustrated in FIG. 3, it is seen that the enzymatic reaction producing gluconic acid extended over a long period of time (about 80 hours), the pH in the reaction tank was consistently held above 4.6, notwithstanding the continuous production of gluconic acid. In fact, the pH actually rose in the reaction tank during the time the data was collected in FIG. 3. Specifically, a five-cell-pair EDI stack 10 as illustrated in FIG. 1 was assembled. Mixed cation-anion resin wafers 12 were used in the stack 10. The resins in the wafers 12 were strong acid cation resins and strong base anion resins with a cation to anion capacity ratio of 1:105. The product of either the enzyme reaction or the fermentation was fed into the reaction compartments 15 and the organic acid produced from the reactions therein was transported into the product compartments 17 by means of the applied current.

Glucose oxidase (GOD), which converts glucose into gluconic acid was selected for an experiment to control the pH in an enzymatic reaction. GOD is sensitive to the pH of the solution and loses most of its activity when the pH drops below 4.0. In the experiment illustrated in FIG. 3, 4 liters of 40 gram/L D-glucose was reacted with 30.5 milligrams of GOD. The solution was pumped into the EDI stack 10 and recirculated

at a flow rate of 300 milliliters per minute. Current was applied to the EDI stack 10, more particularly, across the cathode 20 and anode 22 to remove protons and gluconate ions produced by the enzymatic reaction from compartment 15 to the product compartment 17. FIG. 3 shows the results of the gluconic acid production obtained from the product compartment 17. The pH of the reaction tank was maintained above 4.6 by the inventive EDI process. The reaction was continuously operated for more than 80 hours with reactivity about 0.08 milligrams/milligrams of GOD per minute. Nearly 7 grams per liter of gluconic acid was produced and the separation of the gluconic acid from the reactant approached 100%.

A specialized microorganism that could convert the glucose into lactic acid was used in a fermentation tank in conjunction with the EDI stack 10. 1.0 liters of 1 gram per liter D-glucose reactant with 1.0 gram dry weight whole cells (2.6 gram wet weight whole cells) was circulated between a fermentation tank and the EDI stack 10 at a flow rate of 200 milliliters per minute. Neither growth medium nor nutrients was used or added during the fermentation. FIG. 4 shows the transport of the lactic acid produced from the fermentation in the product compartment 17. 3.0 grams per liter of lactic acid was produced at a reactivity of about 0.3 grams per liter per hour. The separation of lactic acid was nearly 100%. FIG. 5 shows the pH profile of the fermentation tank during the experiment. As noted, the pH of the fermentation was maintained between 5.8 and 7.0 notwithstanding the continuous production of lactic acid.

An example of fine tuning the pH control using the EDI stack is illustrated in FIG. 6. The pH of the solution was manipulated by the applied current and feed flow rate and the concentration of the solution fed into the EDI stack 10. 0.001 normal hydrochloric acid solution was fed into the EDI stack with a 50% cation resin ratio in the resin wafers 12. The EDI stack was operated at two different residence times (RT) for the ion-exchange process, for 40 seconds and 72 seconds. Referring to FIG. 6, it is shown that the general trend of the solution pH could be precisely controlled by adjusting the applied current (i.e., the current utilization).

While there has been disclosed what is considered to be the preferred embodiment of the present invention, it is understood that various changes in the details may be made without departing from the spirit, or sacrificing any of the advantages of the present invention.

Controlled Immobilization of Biocatalytic Enzymes in Separative Bioreactors

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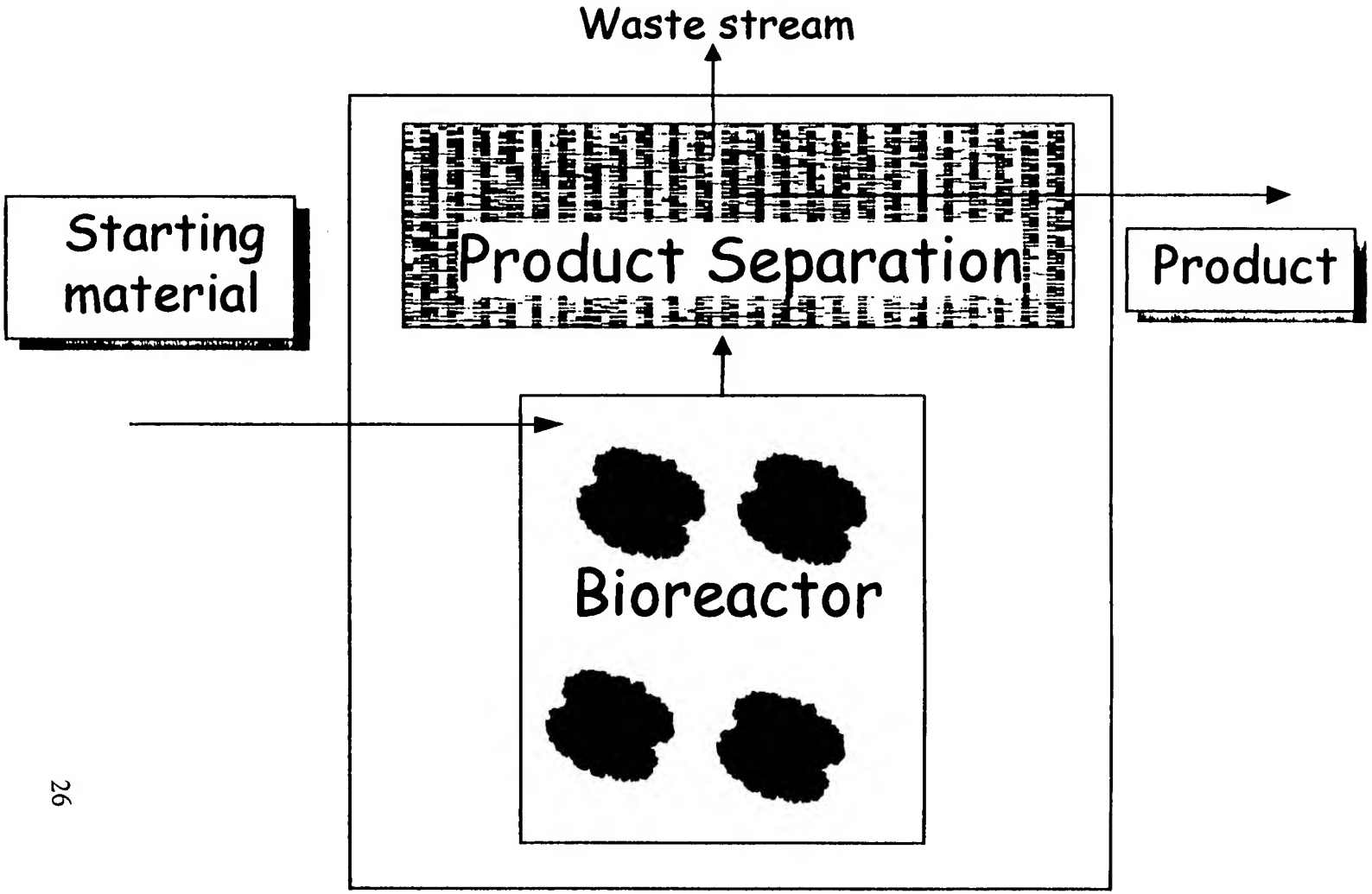
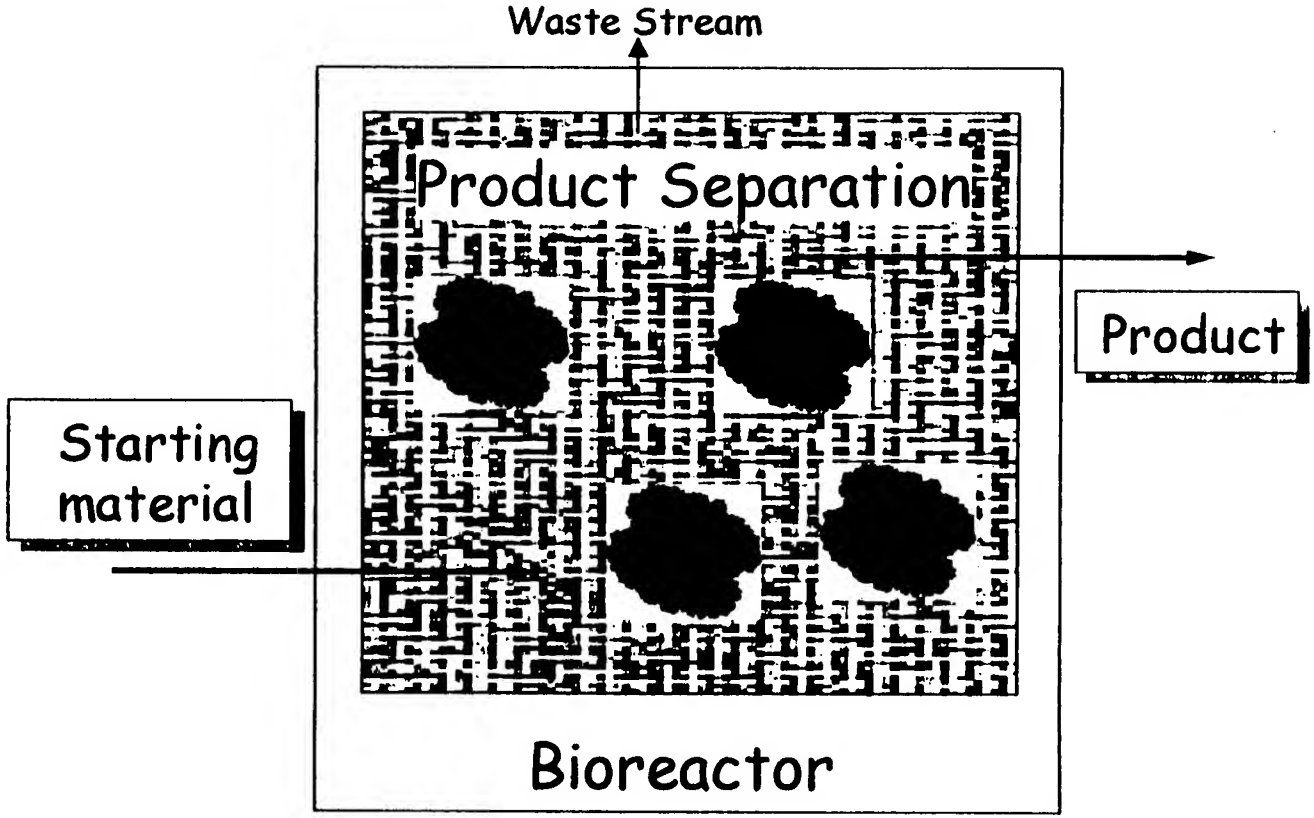
Abstract:

Bioreactions offer an attractive alternative to chemical conversions, because they provide cleaner reactions with fewer by-products and do not require high temperatures and pressures. Bioreactors tend to use microorganisms rather than enzymes for biocatalytic reactions, because of enzyme damage during immobilization and inefficient enzyme regeneration technologies. In this work we address barriers to immobilizing enzymes in a bioreactor. Our separative bioreactor simultaneously produces and separates ionized bioproducts and is based on modified electrodeionization technology (Previsous Patent Application) comprised of a patented porous ion-exchange resin wafer (See Presentation 134g). The enzymes of the separative bioreactor are genetically engineered with a specific "tag" that facilitates chemically-reversible immobilization to a specific "capture" resin. Selective enzyme-capture resins may be incorporated with ion-exchange resins into the porous resin wafer, thus the tagged enzymes are immobilized within the separative bioreactor. The tagged enzyme-capture resin technology provides enzyme-based separative bioreactors with control over three aspects of enzyme immobilization: a chosen site on the enzyme for immobilization (non-destructive enzyme immobilization), targeted capture of the enzyme from whole cells (*in situ* enzyme purification), and control over the removal of enzymes from the bioreactor (*in situ* enzyme regeneration). The added control over enzyme immobilization provides a technology platform for enzyme-based bioreactors of future biorefineries.

Bioreactor and Separation in Two Steps

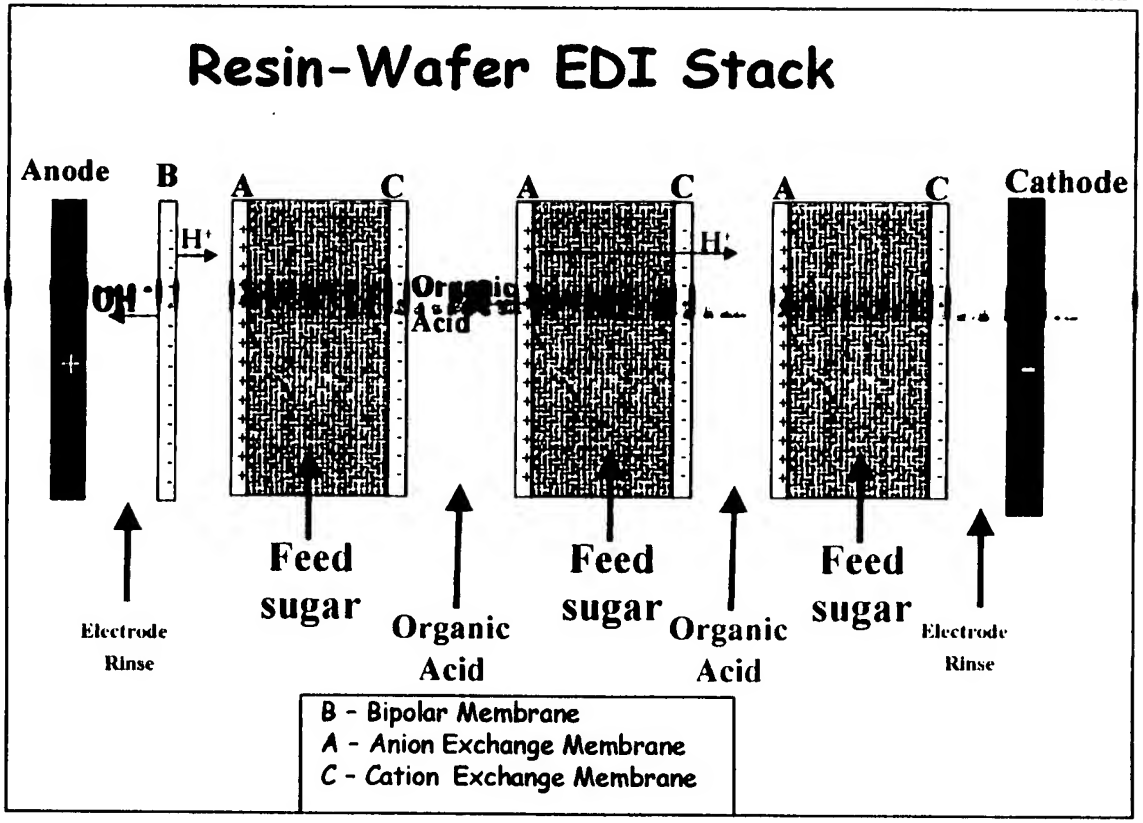


Bioreactor and Separation in ONE Step



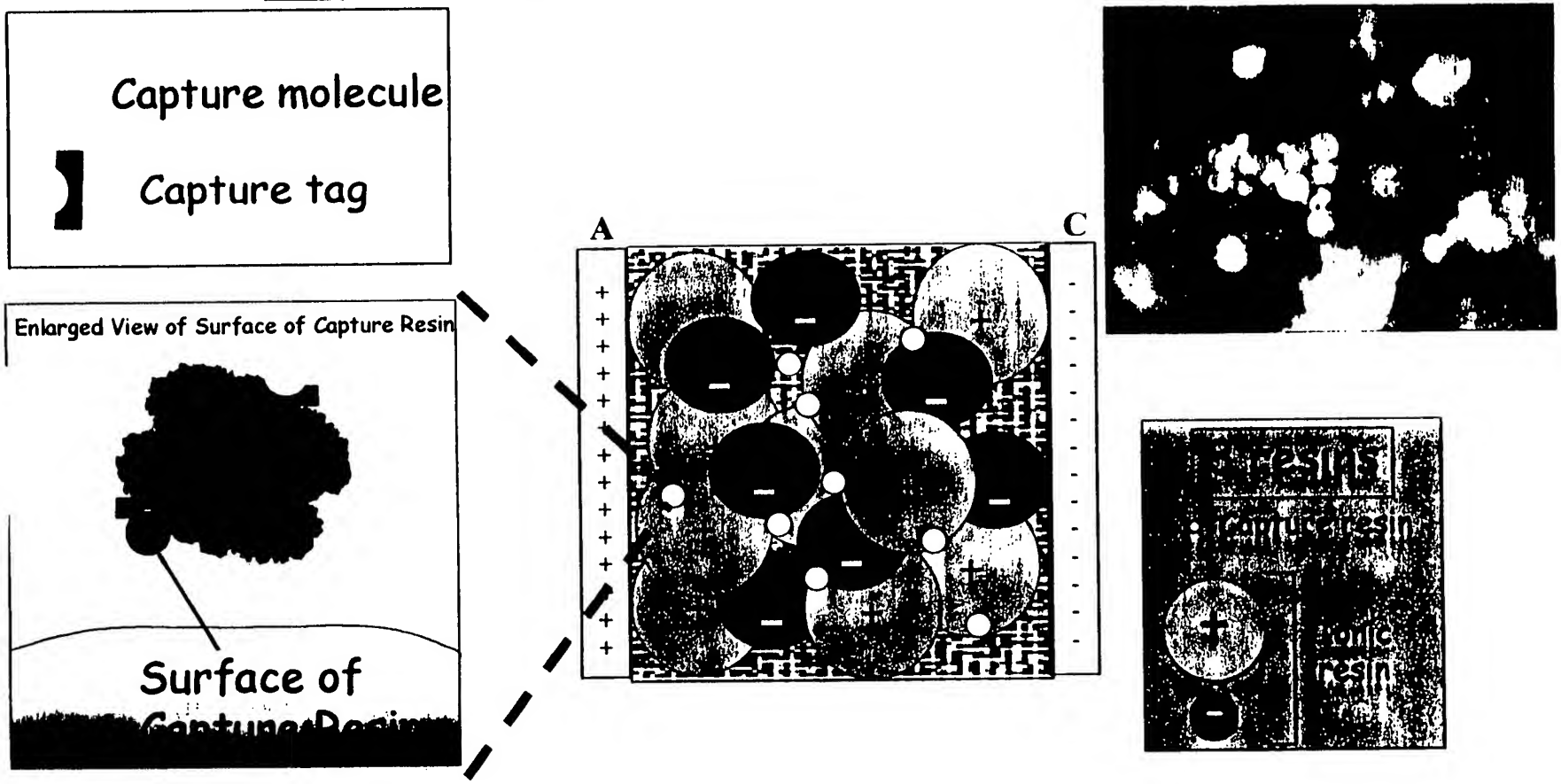
Conventional Bioreaction Process and Separation Process occurs in two steps. ANL combined the Bioreactor and Separation into one to form the **Separative Bioreactor**.

EDI Process -
pH-Controlled EDI Separative Bioreactor



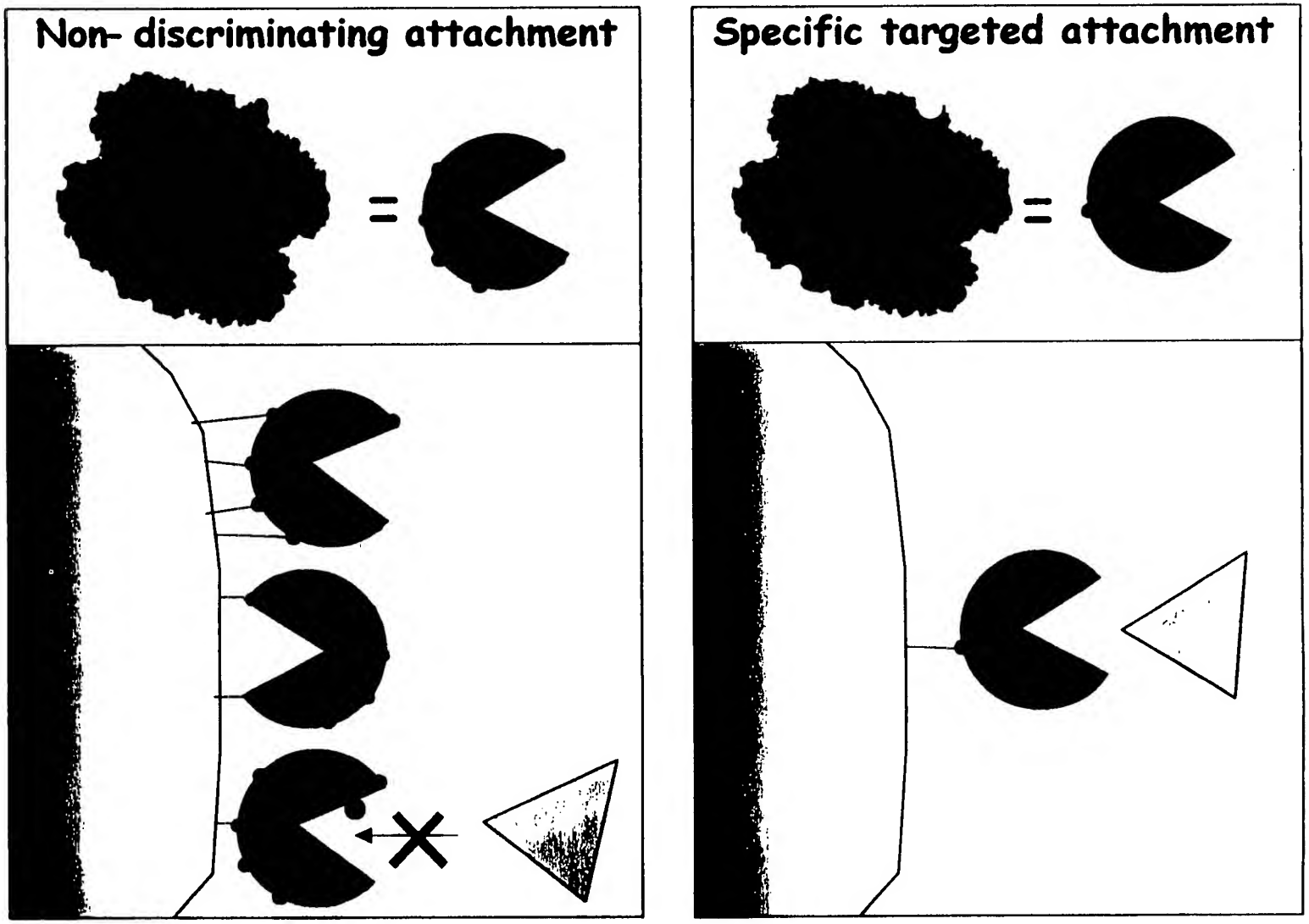
The ANL Separative Bioreactor is a modified EDI technology (Previous Patent Application) composed of a patented resin wafer (contains porous ion exchange resins) sandwiched between ion-exchange membranes placed within an electrical current in an electrodialysis stack. The ANL Separative Bioreactor maintains pH control and separates and purifies charged products efficiently.

Enzyme Immobilized on Capture Resin Within Resin Wafer

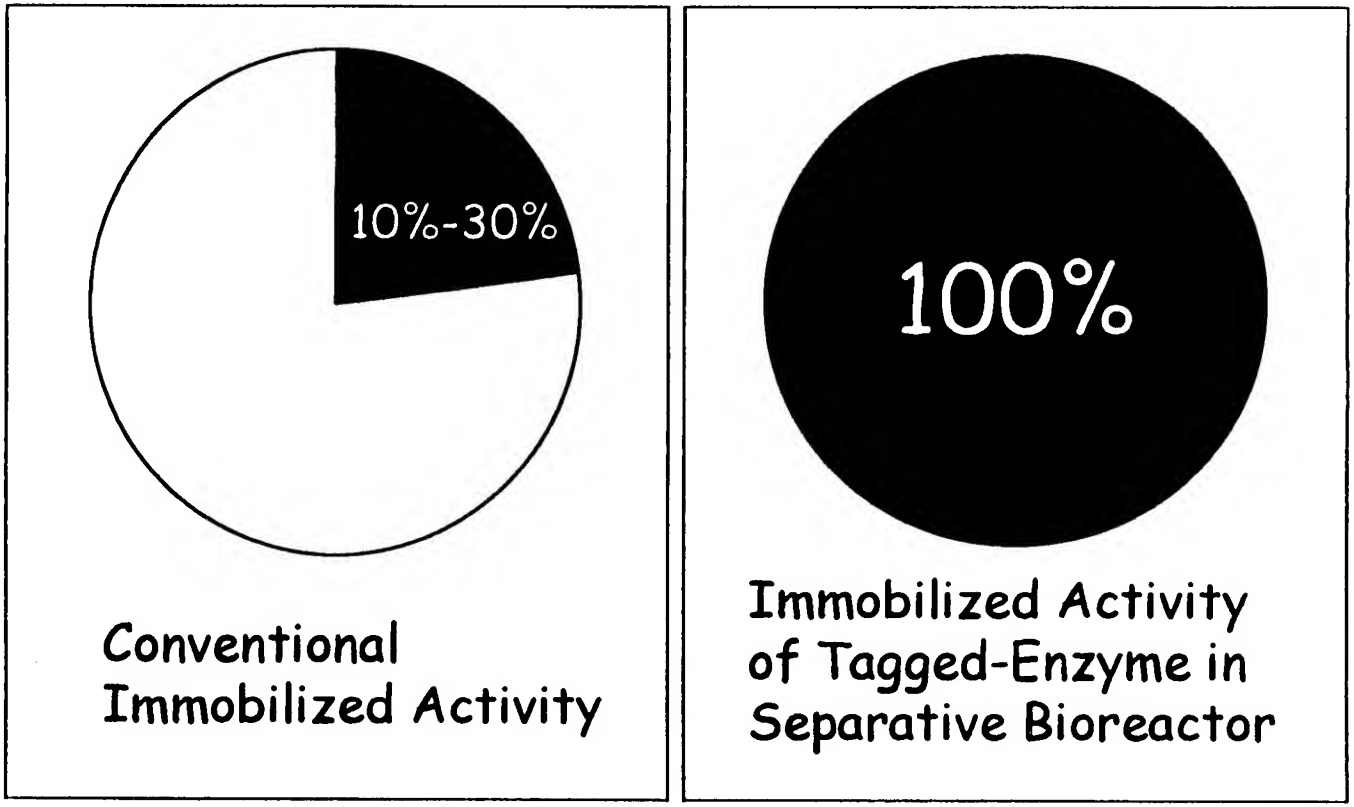


Enzymes are genetically engineered with a tag at either the N-terminus or C-terminus. The tag has affinity for a capture molecule attached to a capture resin. The capture resin is intercalated within the resin wafer of the separative system. Thus, the tagged nzyme is immobilized within the separative system to form the Separative Bioreactor.

Site Specific Immobilization

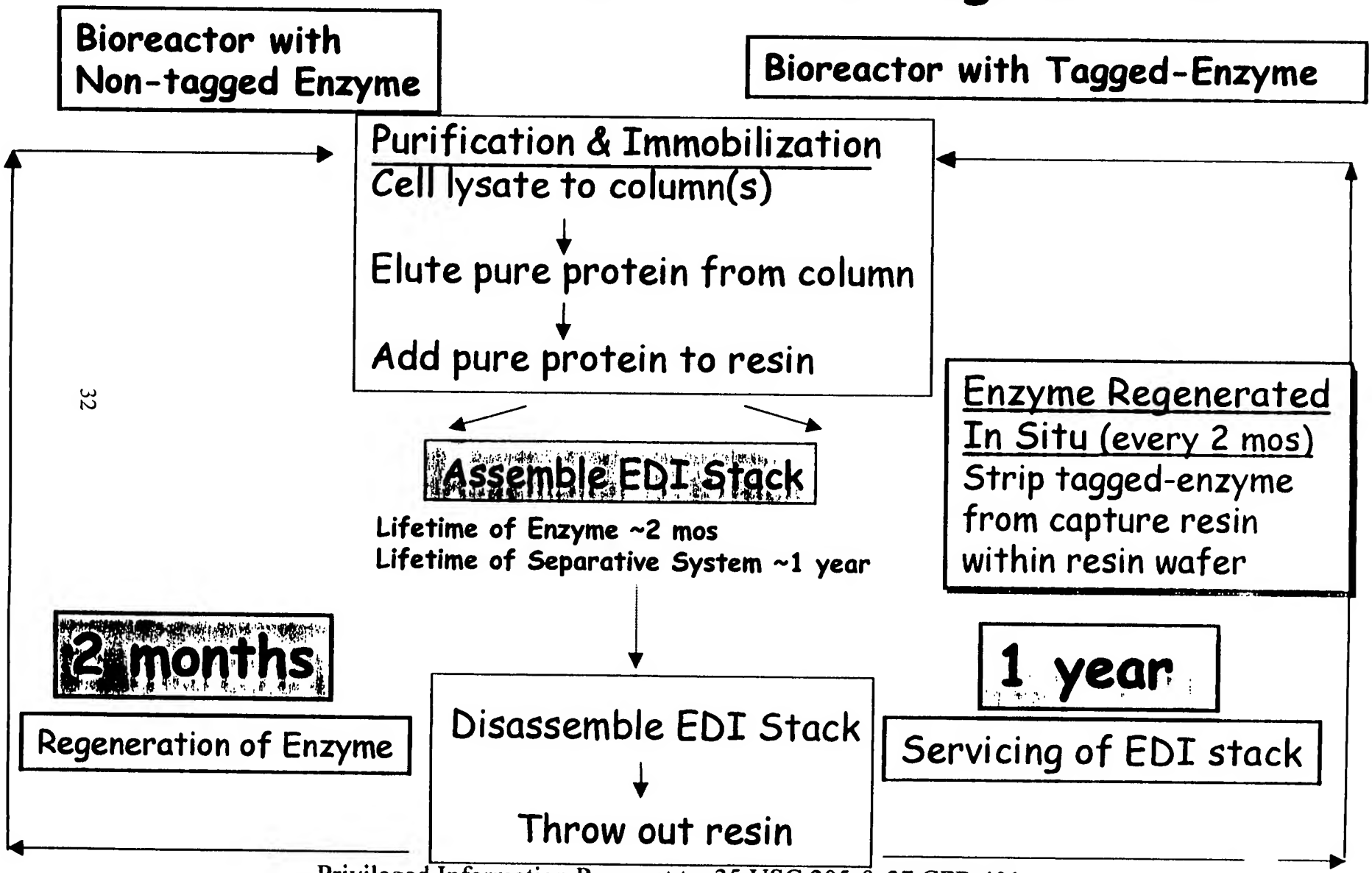


Tagged-Immobilized Activity is Normal



- Site specific immobilization prevents inactivation of the enzyme.
- Normally, accumulation of product inhibits enzyme activity. But in the Separative Bioreactor, immediate product separation prevents product inhibition of the enzyme.

In Situ Immobilization and Regeneration



Binding Capacity and *in situ* Regeneration of Enzyme on Capture Resin in Separative Bioreactor

Reported in %age of binding capacity	Bound	Strip	Rebound
A-tag-Enzyme	100%	~95%	100%
B-tag-Enzyme	25-50%	~50-75%	not tested

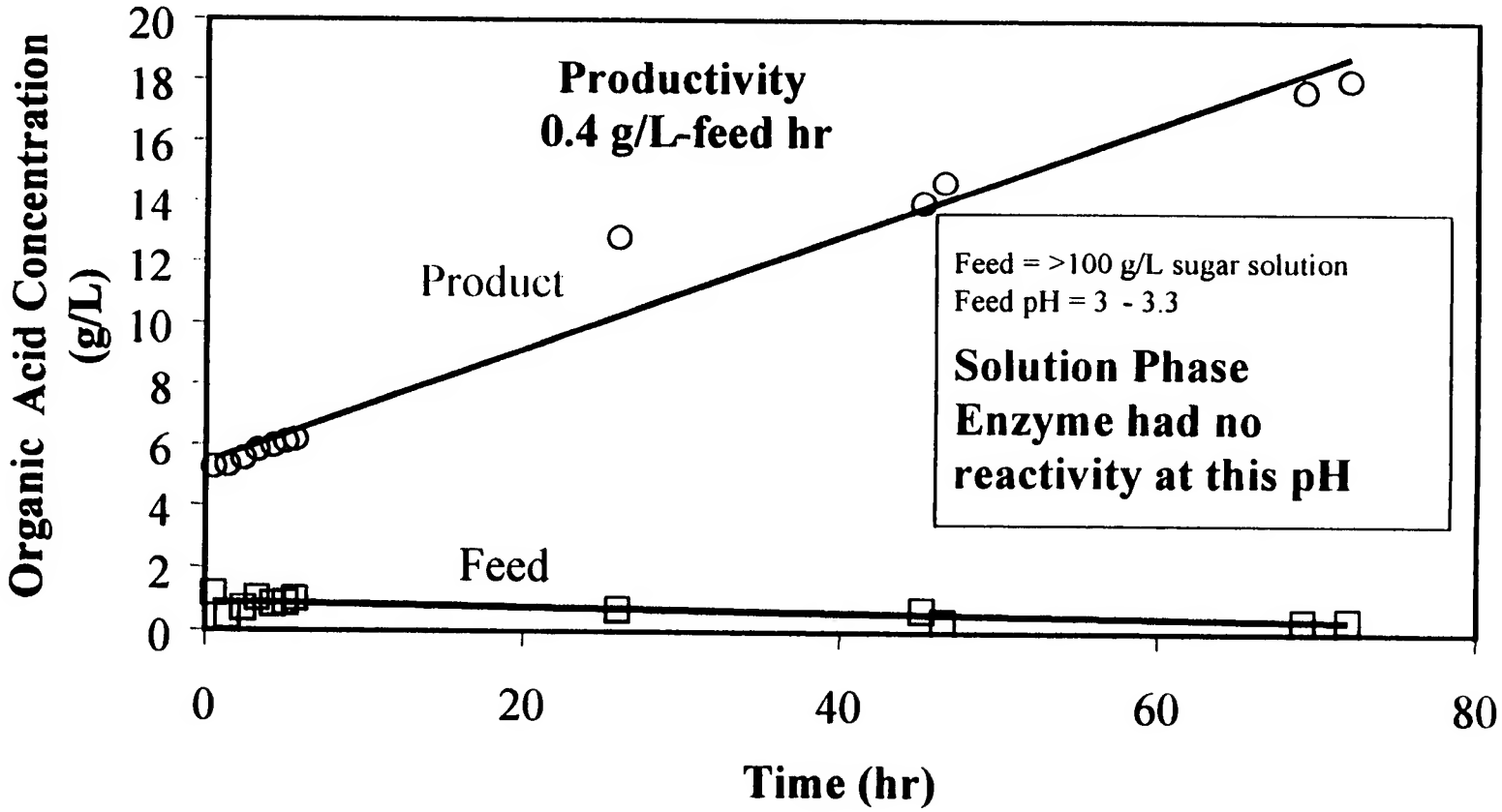
Tagged-enzymes can be washed off by capture molecule homologues, thus enabling *in situ* regeneration. Because of *in situ* regeneration, the Separative Bioreactor needs servicing only once a year, rather than every two months.

Conclusions

Using tagged enzymes in the separative bioreactor process offers:

- Specific immobilization in which immobilized enzymes retain normal activity.
- *In situ* regeneration, inactive enzyme is removed *in situ* and fresh tagged-enzyme is added *in situ*, without having to disassemble and reassemble the separative bioreactor system.

Gluconic Acid Transport with Immobilized Tagged-Enzyme in Separative Bioreactor



In the Separative Bioreactor, organic acid product is simultaneously separated and produced.

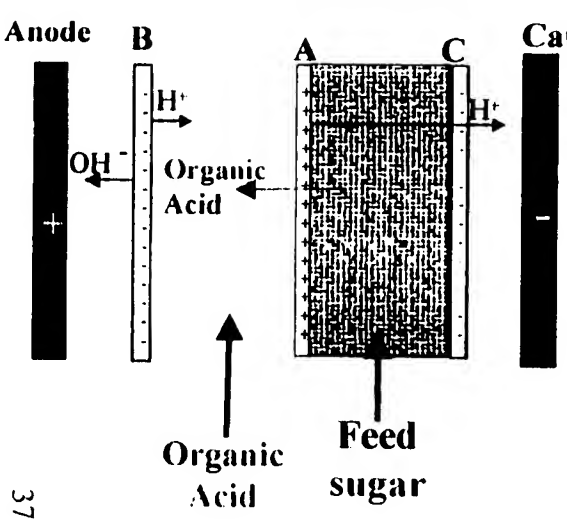
Immobilized Biocatalytic Enzymes in Electrodeionization

ANL-IN-02-057

Presented by
Edward St.Martin, Yupo Jim Lin, Jamie Hestekin,
Michelle Arora, Seth Snyder
Chemical & Biological Technology Section
Energy Systems Division

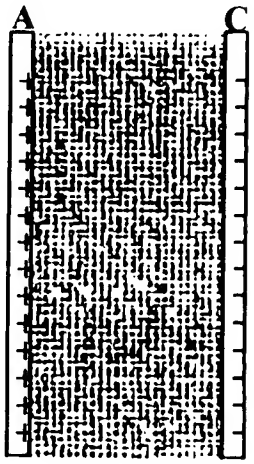
To
Intellectual Property Decision Group
October 25, 2002

Electrodeionization (EDI) Intellectual Property Package



Patent 1

EDI resin wafer



Resin Wafer
Enclosed non-loose resin, no leakage

Patent 2

EDI process

EDI Separative Bioreactor
pH maintained without buffer
Controlled ratios of Anionic/Cationic resin-dictate pH
Simultaneous product formation and product removal

Patent 3

Specific Enzyme Immobilization

Tagged Enzyme for Specific & Economic Immobilization in EDI Separative Bioreactor

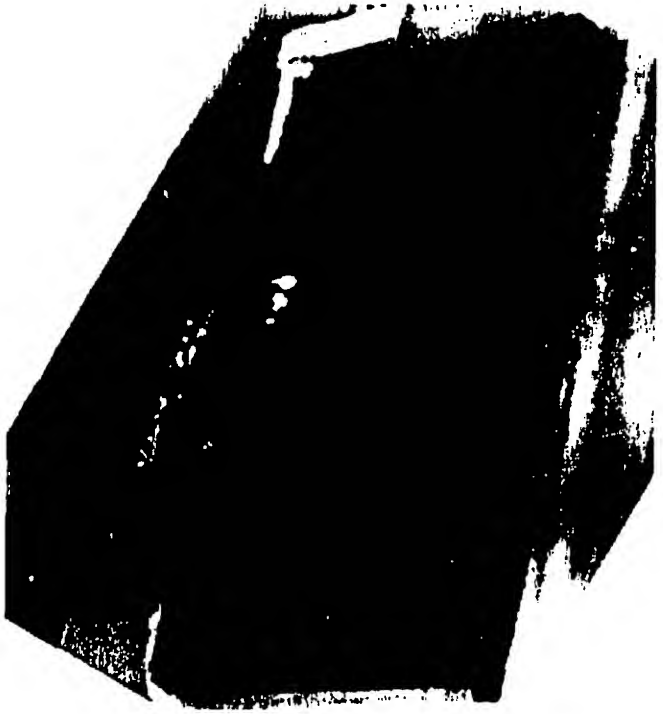
Utility:
3rd capture resin in Resin Wafer
Specific immobilization of enzyme
Regeneration of enzyme in Resin Wafer

EDI Resin Wafer

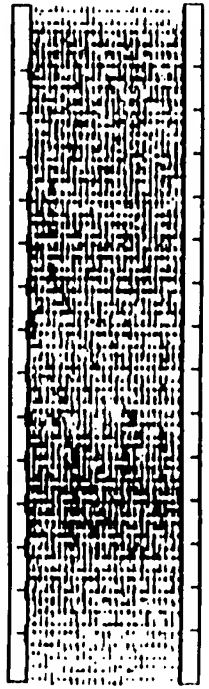
Leaky Technology: Loose Resin

Contained Technology: Resin Wafer

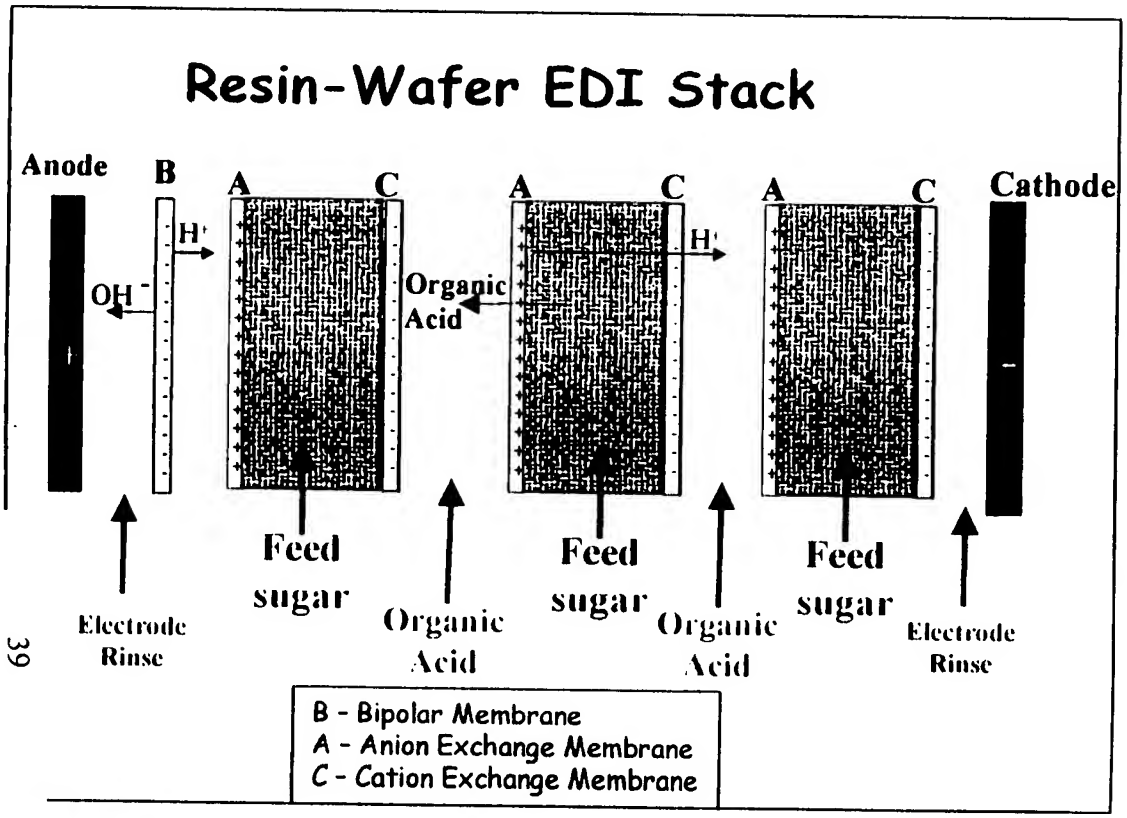
Actual

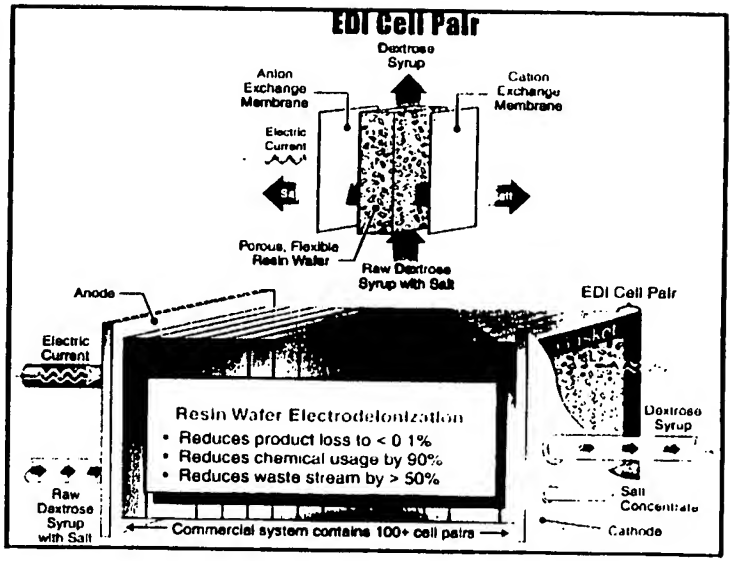


Schematic

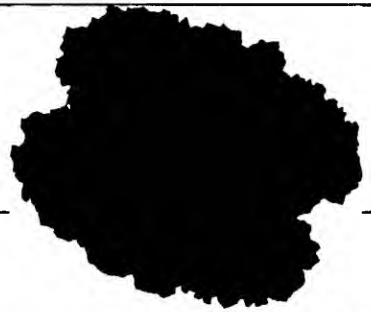


EDI Process -
pH-Controlled EDI Separative Bioreactor





Specific Enzyme Immobilization -
Glucose Fructose Oxidoreductase (GFOR)

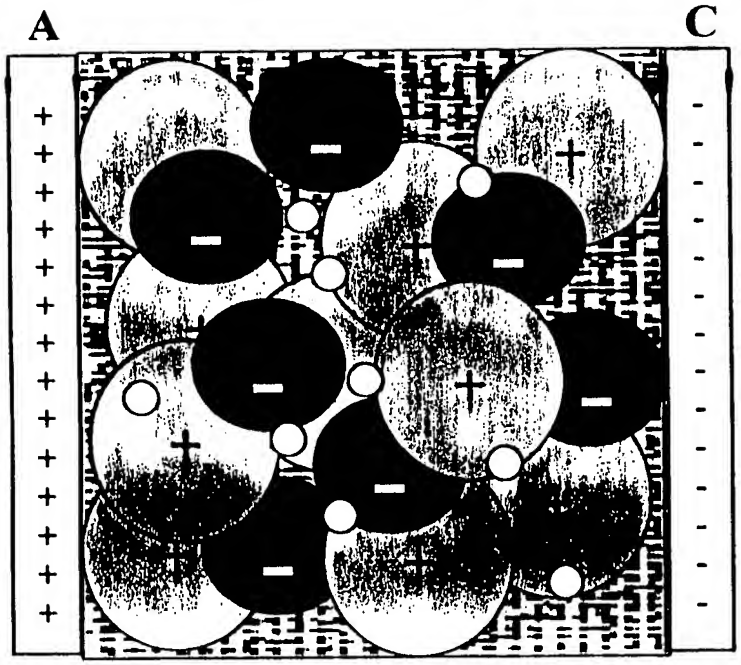


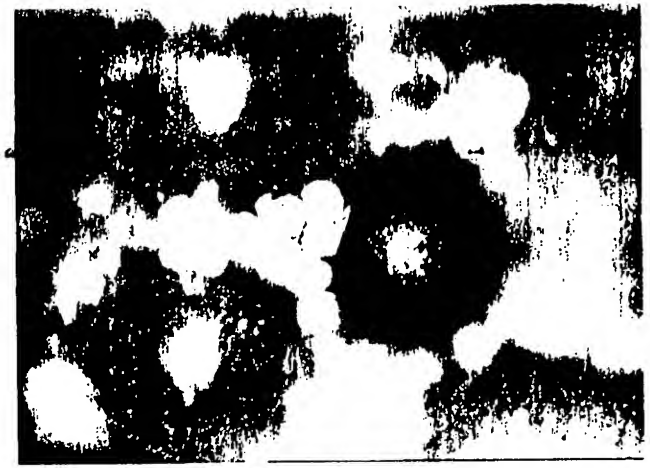
Glucose → Gluconolactone → Gluconate

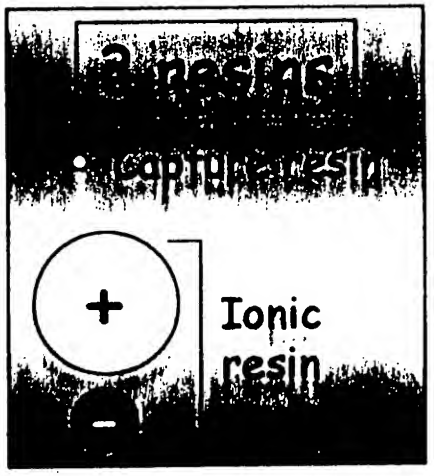
NADP+ → NADPH

Sorbitol ← Fructose

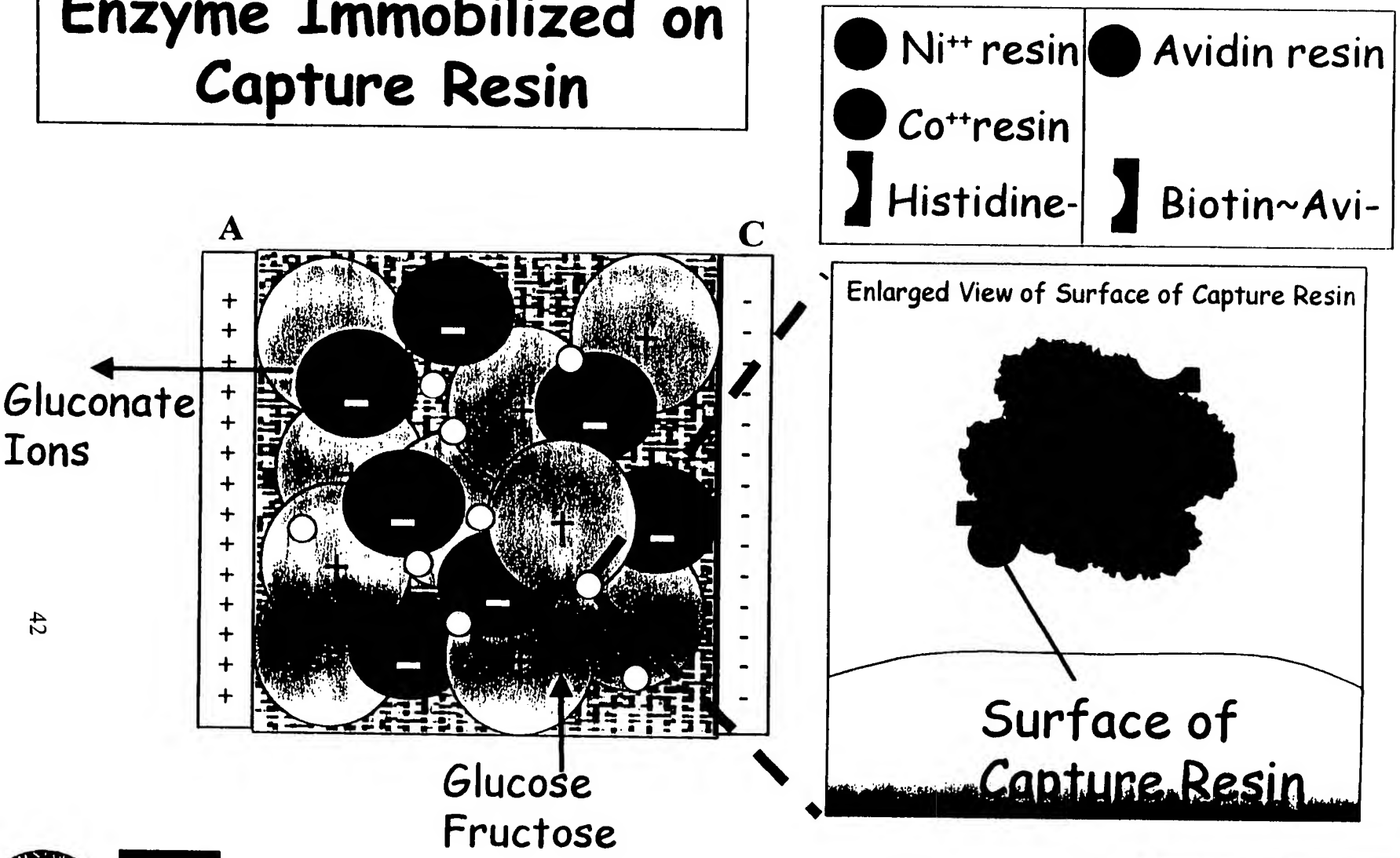
EDI Three Resin Wafer Contains Capture Resin



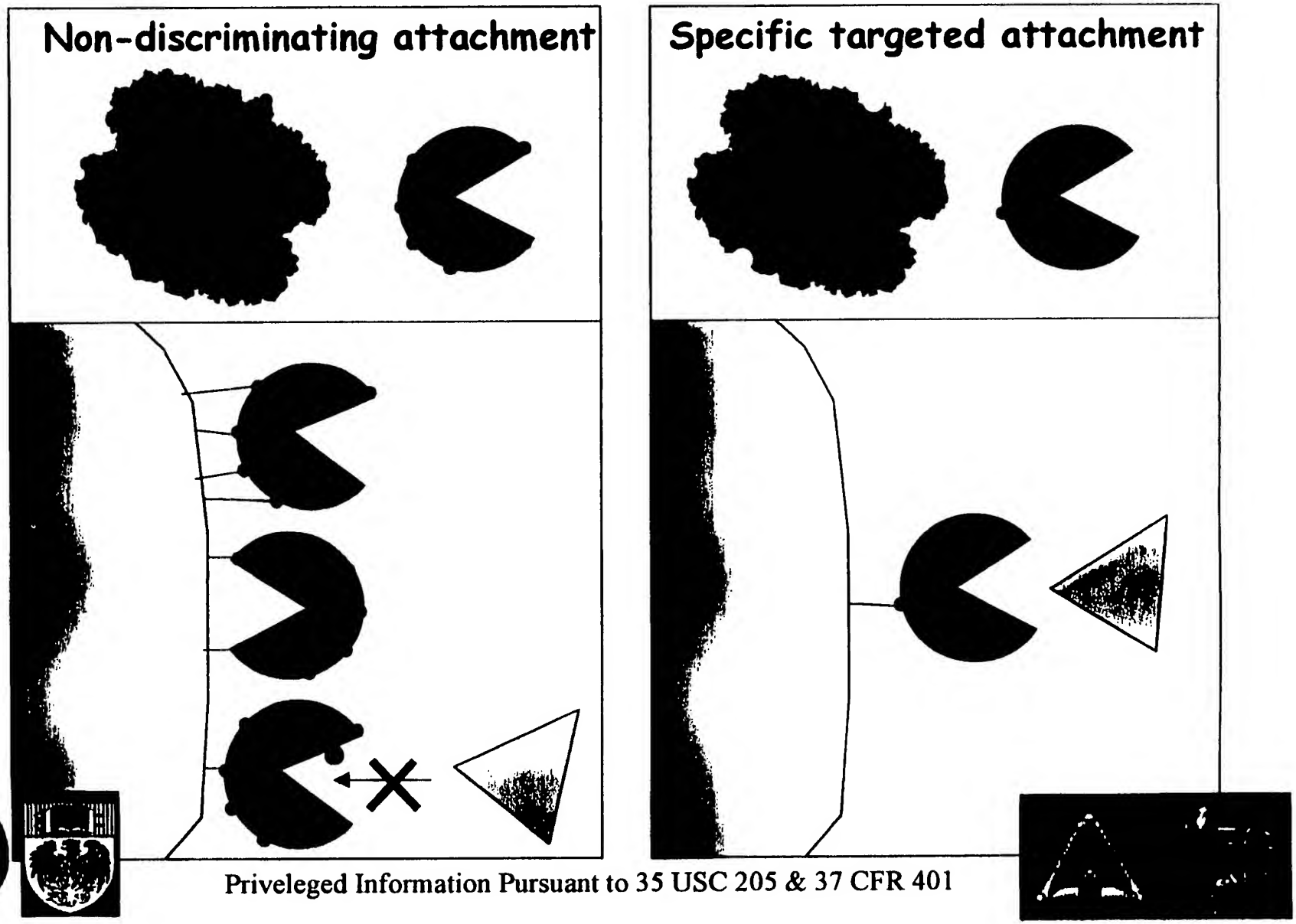




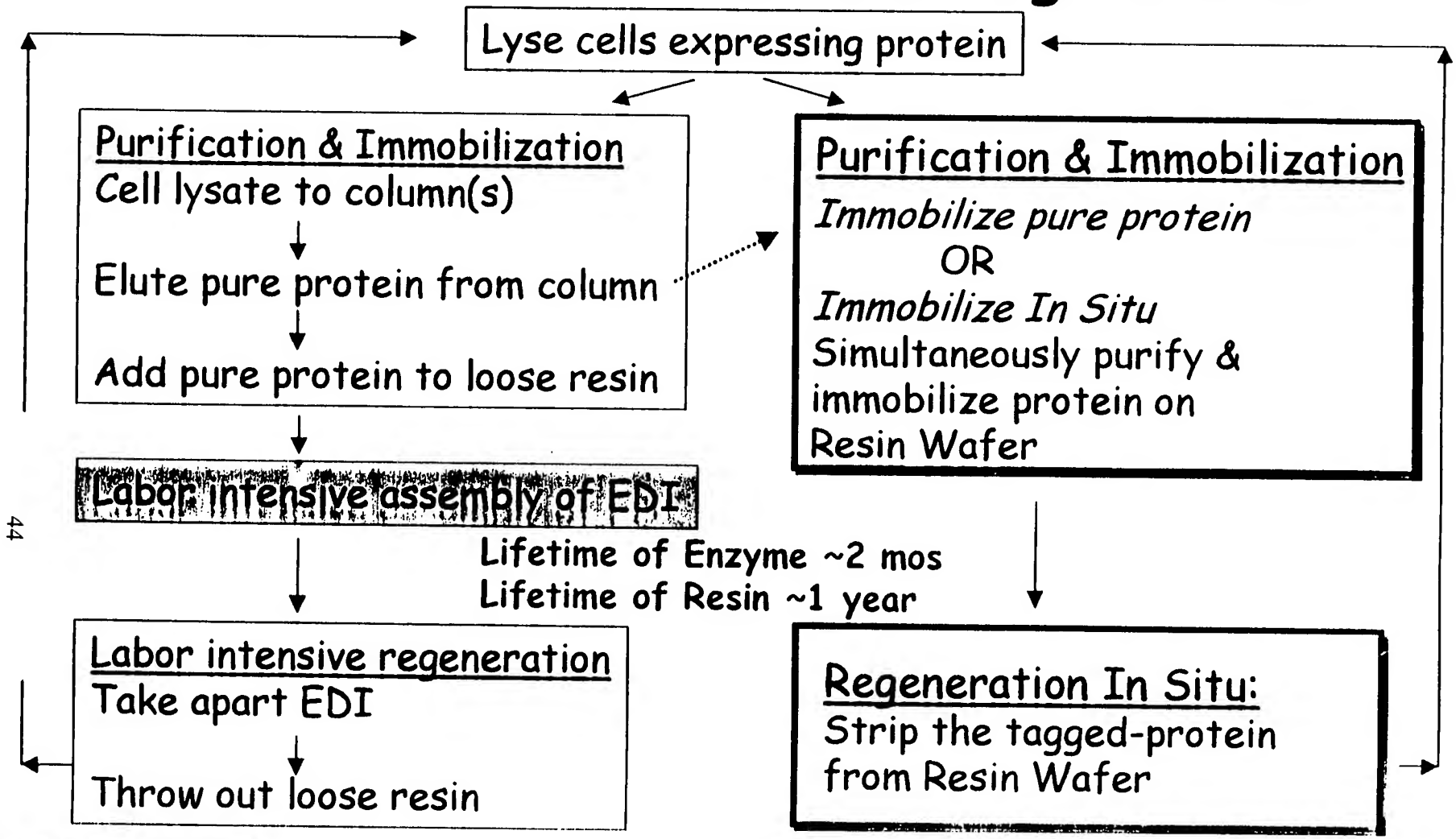
Enzyme Immobilized on Capture Resin



Specific Immobilization



In Situ Immobilization and Regeneration



Capture Resin Binding Capacity is Normal in Resin Wafer

Ni^{++} Capture Resin

·Capture resin in Resin Wafer Retains ~100% of binding capacity

His-GFOR immobilized 12-20ug/ul Resin binding capacity: 5-10 mg/ml (300-500 nmol @ ~20 kDa), or 2.5 - 5 mg/ml (150-250 nmol) for ~40 kDa

Avidin Capture Resin

·Capture resin in Resin Wafer Retains ~25-50% of binding capacity (bead size ~40% smaller than Ni-resin)

Biotin~Avi-GFOR immobilized 0.2 ug Avi-GFOR/ul Resin binding capacity: 25-40nmol (0.4 – 0.8 mg) biotinylated cytochrome C (~38 kDa) per ml resin

Regeneration of Enzyme on Capture Resin in Resin Wafer

Reported in %age of binding capacity	Bound	Strip	Rebound
His-GFOR	100%	~95%	10%
Biotin~Avi-GFOR	25-50%	~50-75%	not tested

Immobilized Activity is Normal

Resin Wafer :
His and Avi GFOR:
- **Activity normal** when immobilized on capture resin in resin wafer without buffer
- Activity for a limited time (eventually low pH and gluconic acid accumulation kills GFOR activity)

Resin Wafer in EDI:
His-GFOR:
- **Activity normal** when on capture resin in resin wafer without buffer *in EDI electrolysis*
- Activity lasts for days (because pH kept constant and gluconic acid separated).